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Quinone-Adducted Proteins as Potential Biomarkers of  
Breast Cancer Risk

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13. ABSTRACT (Maximum 200 Words)

The purpose of this research is to develop a specific monoclonal antibody (MAb) for intracellular detection and quantification of E<sub>2</sub>-3,4-Q adducted to proteins, as a potential biomarker of breast cancer risk. An established, protein-coupling chemistry was applied successfully to production of an immunogen, via linkage of E<sub>2</sub>-3,4-Q to cationized protein, through a short linker. A coating complex for indirect ELISA and a low molecular-weight conjugate for competitive ELISA were prepared by spontaneous adduction of E<sub>2</sub>-3,4-Q to an unmodified protein and a short-chain molecule with a primary amino group, respectively. ELISA screening identified a hybridoma producing relatively large amounts of MAb with high signal-to-noise ratio. The affinity constant (0.5 x 10<sup>8</sup> M<sup>-1</sup>) for binding to E<sub>2</sub>-3,4-Q was determined by competitive ELISA. In specifically designed immunohistochemical (IHC)-staining protocols, the antibody produced intense and specific staining with no detectable cross-reactivity to E<sub>2</sub>-2,3-Q and little or no background. Failure to detect IHC staining in paraffin sections of ACI rat breast tumors or human breast tumors is associated with loss of the epitope during microwave recovery or formalin fixation. Optimization of tissue fixation is required for validation studies that can be based on Quantitative Fluorescence Imaging Analysis or Surface-Enhanced Laser Desorption Ionization/tandem mass spectrometry.

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## INTRODUCTION

Breast cancer, the leading cause of cancer-related deaths among non-smoking women, is linked to cumulative estrogen exposure. A compelling body of information supports a close association between estrogen-dependent carcinogenesis (E-DC) and preferential 4-hydroxylation of 17 $\beta$ -estradiol ( $E_2$ ) to form 4-OH $E_2$ , in both model systems and human breast tissue (1). Formation of  $E_2$ -3,4-quinone ( $E_2$ -3,4-Q) the oxidation product of 4-OH $E_2$  is thought to be essential for E-DC of the breast and can contribute to E-DC *via* indirect or direct damage to DNA. In tissues susceptible to E-DC, 4-OH $E_2$  and  $E_2$ -3,4-Q may escape detoxification, *e.g.*, methylation of the catechol or glutathione conjugation of the quinone, and form  $E_2$ -3,4-Q adducts to proteins. The presence of these adducts in nipple aspirate or fine-needle aspirate cells may serve as a biological marker of non-detoxified  $E_2$ -3,4-Q and breast cancer risk. These considerations have prompted the present proposal aimed at a) producing a monoclonal antibody (MAb) specific for  $E_2$ -3,4-Q adducted to any protein, and b) evaluating its immunochemical and immunohistochemical characteristics.

## BODY

A. Synthesis of an immunogen consisting of estradiol-3,4-quinone ( $E_2$ -3,4-Q) covalently linked to hen egg albumin (OA)

1. Basis for the idea that  $E_2$ -3,4-Q could form simple and stable adducts to protein amino groups

The literature contains no published studies of either spontaneous adduction or chemical coupling of  $E_2$ -3,4-Q to proteins. The possibility of spontaneous formation of simple and stable adducts of  $E_2$ -3,4-Q to amino side groups (primarily  $\epsilon$ -amino groups of lysine residues) of proteins was conceived in light of 1) the electrophilic nature of carbon-1 of  $E_2$ -3,4-Q and its reactivity with mimics of amino acid side chains (2), and 2) published studies of spontaneous adduction of urushiol quinones to human serum albumin and 1-aminopentane a model primary amine (3). Liberato *et al.* (3) demonstrated regiospecific attack of 1-amino-pentane at carbon-5 of 3-heptadecylbenzoquinone, producing the aminoquinone, 3-heptadecyl-5-(pentylamino)-1,2-benzoquinone (**Figure 1**). The reaction produced a characteristic red shift in the absorption spectrum of the quinone. The aminoquinone had an absorption maximum of 480 nm and exhibited a distinctive red color. The authors concluded, that urushiol quinones (with configurations analogous to the estrogen quinones) react efficiently with primary amino groups of HSA

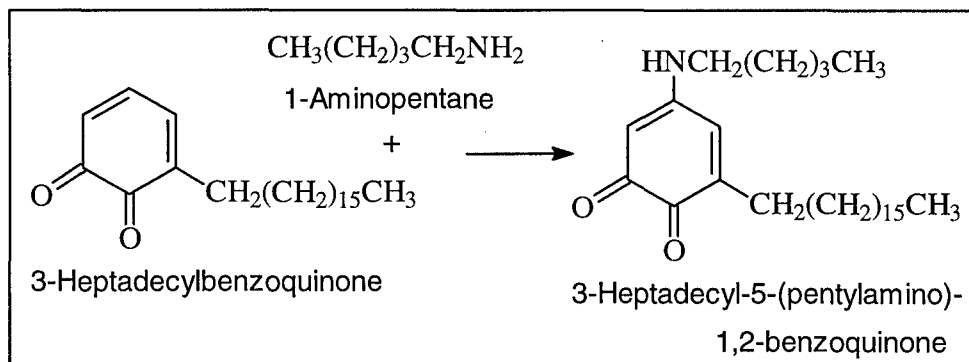


Figure 1

thus producing aminoquinones represented by 3-heptadecyl-5-(pentylamino)-1,2-benzoquinone.

The work of Liberato *et al.* (3) prompted a study of the reaction of E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q with N-acetyl-L-lysine (N-AcLys) in acetic acid/water (4). E<sub>2</sub>-3,4-Q a yellow chromophore reacted efficiently with N-AcLys, producing estradiol-3,4-quinone-1-N-acetyl-L-lysine (E<sub>2</sub>-3,4-Q-1-N-AcLys) with a 73% yield (**Figure 2**). Genesis of E<sub>2</sub>-3,4-Q-1-N-AcLys in the reaction mixture produced a characteristic red shift in the absorption spectrum of the quinone. The aminoquinone exhibited a distinctive red color that permits convenient monitoring of the occurrence and progression of the reaction and quantification of the aminoquinone.

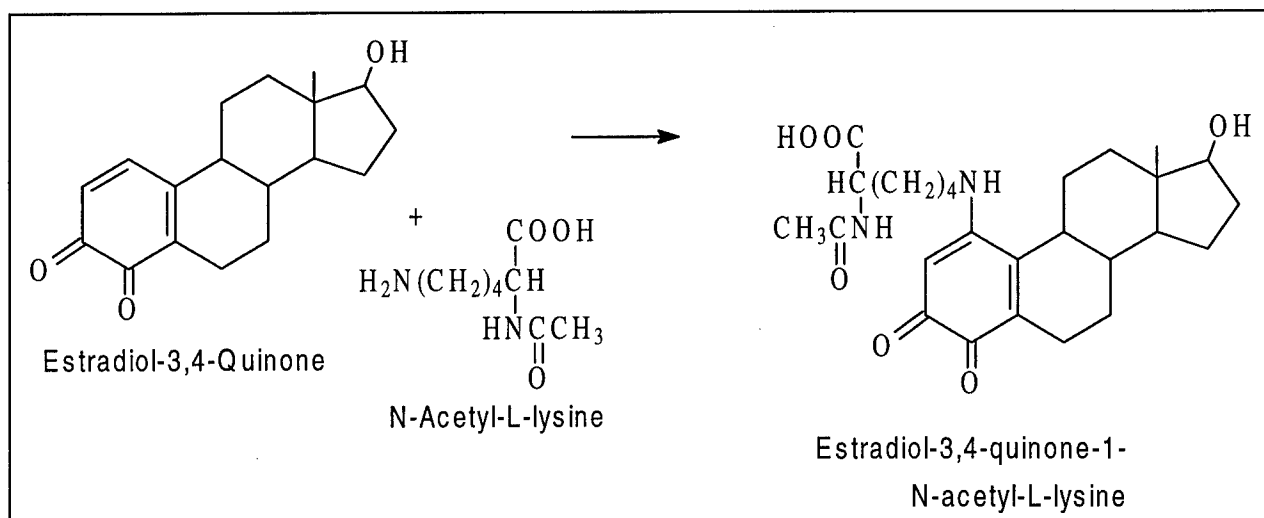


Figure 2

Reaction of E<sub>2</sub>-2,3-Q with N-AcLys produced 2-hydroxyestradiol-6-N-acetyl-L-lysine (2-OHE<sub>2</sub>-6-N-AcLys) with a 38% yield (**Figure 3**). The latter is not a chromophore, consequently the yellow color (due to E<sub>2</sub>-2,3-Q) of the reaction mixture faded as the reaction proceeded.

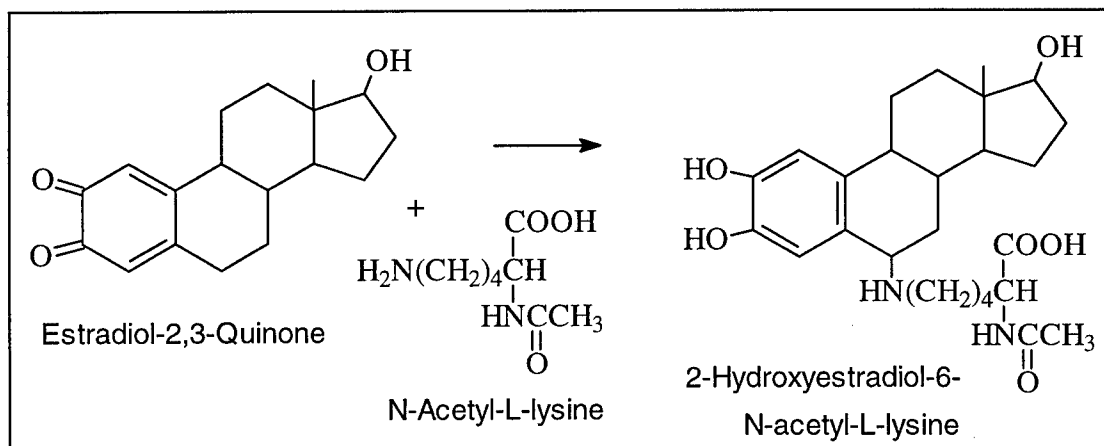


Figure 3

This work demonstrated the rather clean reaction of E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q with primary amino groups in acetic acid/water and supported the possibility of direct adduction of the estrogen quinones with proteins and development of a monoclonal antibody that could easily discriminate between amino-adducted E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q. Significant questions, however, remained unanswered. Does E<sub>2</sub>-3,4-Q form adducts to primary amino groups of model compounds and to proteins, at or near physiological pH? Does the reaction of E<sub>2</sub>-3,4-Q with primary amino groups occur efficiently, only in highly acidic environments? Does direct adduction of E<sub>2</sub>-3,4-Q to proteins occur at a number of sites sufficient to produce an effective immunogen needed for induction of anti-E<sub>2</sub>-3,4-Q specific antibodies, and a coating complex needed for immunochemical studies of antibody potency and specificity? Do steric limitations posed by fixed positioning of reactive nucleophiles (*e.g.*, primary amino groups) in relation to other amino acid residues on proteins disfavor useful or spontaneous adduction of E<sub>2</sub>-3,4-Q?

2. Principles guiding production of an immunogen that would induce antibodies specifically reactive with stable adducts of E<sub>2</sub>-3,4-Q to proteins or other macromolecules

a. A low-molecular-weight protein [hen egg albumin (OA)], in comparison to a high molecular-weight protein, *e.g.*, keyhole limpet hemocyanin (KLH), is a superior carrier for immunization against E<sub>2</sub>-3,4-Q. The investigator has determined in previous studies that sufficient substitution of KLH with a hydrophobic hapten, such as E<sub>2</sub>-3,4-Q, produces an unmanageable paste. Limiting the rate of haptenation of KLH will produce an immunogen that may elicit anti-E<sub>2</sub>-3,4-Q clones as a small minority against the background of a wide diversity of clones against relatively potent KLH-specific epitopes. In contrast, high-frequency haptenation of OA with a hydrophobic hapten will produce a manageable precipitate that can be washed and resuspended in buffer. The fine precipitate acts as an immuno-adjuvant and at the same time, relevant epitope (E<sub>2</sub>-3,4-Q) frequency is high.

b. The immunogen must be produced by either direct adduction of E<sub>2</sub>-3,4-Q to protein or coupling *via* a short linker. Preliminary studies demonstrated spontaneous formation of E<sub>2</sub>-3,4-Q adducts to both OA and bovine serum albumin (BSA) in phosphate buffer at pH 6.5. Thus, supporting the feasibility of spontaneous adduction of endogenously generated E<sub>2</sub>-3,4-Q to cellular proteins. These adducts will be in close proximity to the protein surface and are expected to be recognized by antibodies whose binding "pockets" are relatively shallow. Immunization of mice with immunogen formed by either direct adduction of E<sub>2</sub>-3,4-Q or coupling *via* a short linker will favor induction of efficient antibodies with "shallow" binding sites. The use of long-arm linkers would allow induction of antibodies with deep, binding "pockets" that may be sterically hindered in their approach to adducts formed spontaneously on cellular proteins.

c. The immunogen will induce antibodies that recognize E<sub>2</sub>-3,4-Q but not E<sub>2</sub>-2,3-Q. Carcinogenesis studies support E<sub>2</sub>-3,4-Q as the principal carcinogen inducing the majority of breast cancers. Both E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q can be formed in breast epithelial cells. The corresponding catechols (4-OHE<sub>2</sub> and 2-OHE<sub>2</sub>) have been detected in normal and cancerous breast tissue from women, however, 4-OHE<sub>2</sub> the precursor to E<sub>2</sub>-3,4-Q is more abundant in cancerous breast tissue. The reactions of E<sub>2</sub>-3,4-Q and E<sub>2</sub>-2,3-Q with primary amino groups (abundant nucleophiles of proteins) assure that antibodies induced against E<sub>2</sub>-3,4-Q adducted to protein will react poorly if at all with E<sub>2</sub>-



2,3-Q adducted to protein (**Figure 4**). Linkage at carbon-1 of E<sub>2</sub>-3,4-Q (represented by E<sub>2</sub>-3,4-Q-1-N-GABA) and at carbon-6 of E<sub>2</sub>-2,3-Q (represented by 4-OHE<sub>2</sub>-6-N-GABA) produce markedly different molecular configurations these adducted quinones present to receptors of the immune system (**Figure 4**). This is in contrast to potential cross-reactivity of induced antibodies with free E<sub>2</sub>-2,3-Q, which is of no consequence for immunohistochemical detection of protein-adducted E<sub>2</sub>-3,4-Q. Free catechols and quinones are expected to be present at very low concentrations (a few pmols per mg tissue) and will be removed during processing of tissues and cells for immunohistochemistry.

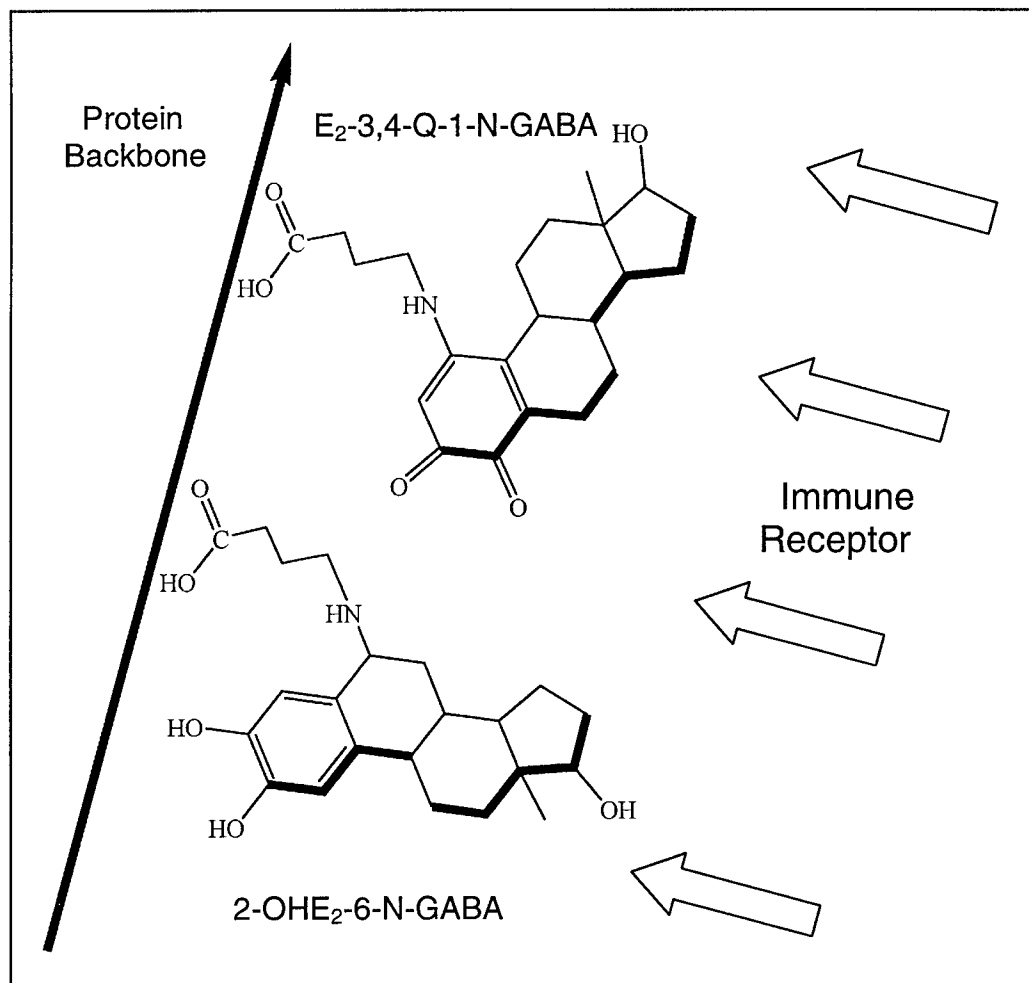


Figure 4

### 3. Synthesis I: Direct coupling of E<sub>2</sub>-3,4-Q to hen egg albumin (OA)

#### a. Preparation of E<sub>2</sub>-3,4-Q

E<sub>2</sub>-3,4-Q was prepared from 4-hydroxyestradiol (4-OHE<sub>2</sub>) by standard procedures, immediately prior to use. Three milligrams (mg) of 4-OHE<sub>2</sub> were dissolved in 3.0 milliliters (mL) of acetonitrile (CH<sub>3</sub>CN) by sonication. This produced a clear and colorless solution. Sodium acetate buffer (pH 6.5) and manganese oxide (MnO<sub>2</sub>) were added to the solution and the mixture was vortexed every minute (min) for 10 min. The

mixture was filtered with a 0.45  $\mu$  PTFE filter and the clear yellow filtrate ( $E_2$ -3,4-Q dissolved in  $CH_3CN$ ) was transferred to a 25 mL rotary evaporator flask. Dimethylformamide (DMF, 1.5 mL) was added to the flask and mixed with the  $E_2$ -3,4-Q solution.  $CH_3CN$  was evaporated off by rotary evaporation leaving  $E_2$ -3,4-Q dissolved in DMF/acetate buffer.

b. Coupling  $E_2$ -3,4-Q to OA

$E_2$ -3,4-Q was directly linked to OA, under conditions developed as part of the present research initiative. Procedures for direct coupling of  $E_2$ -3,4-Q are not published in the scientific literature. A solution of 72 mg OA dissolved in 20 mL phosphate buffer at pH 6.5 (PB6.5) was dialyzed against PB6.5. Three-hundred microliters ( $\mu$ L) of  $E_2$ -3,4-Q solution was added to each 2.5 mL of dialyzed OA and the reaction mixture was stirred for 15-20 hours (hr) at room temperature. The clear reaction mixture changed from a yellow color to a characteristic red color that indicates coupling of the quinone to the protein. Non-reacted  $E_2$ -3,4-Q was extracted into cold acetone and the precipitated protein was dried and stored as a dry powder under vacuum. A solution produced by dissolving a small quantity of dried product in phosphate buffered saline, exhibited a characteristic red color indicative of adducted  $E_2$ -3,4-Q.

c. Estimation of the degree of substitution

The reaction between  $E_2$ -3,4-Q and a large, molar excess of N-acetyl-L-lysine (N-AcLys) served as a reference reaction for the reaction between  $E_2$ -3,4-Q and OA. Change in the color of both reaction mixtures was the same and the reaction products had the same absorption maximum at 510 nm. Assuming quantitative coupling of  $E_2$ -3,4-Q to N-AcLys, the aminoquinone had an extinction coefficient (in buffer, at 510 nm) of  $6.55 \times 10^4$  M. The molar ratio of  $E_2$ -3,4-Q/OA calculated with this coefficient was 2:1.

4. Synthesis II: Coupling  $E_2$ -3,4-Q to OA *via* the linker gamma-aminobutyric acid (GABA).

In this procedure,  $E_2$ -3,4-Q was coupled to gamma-aminobutyric acid (GABA) and then the  $E_2$ -3,4-Q-GABA complex was linked to amino side groups of cationized OA (catOA) in a reaction catalyzed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxy-sulfosuccinimide (S-NHS). The conditions of this procedure were developed as part of the present program and this coupling of  $E_2$ -3,4-Q to protein is unique. Procedures for coupling  $E_2$ -3,4-Q to protein *via* a linker are not published in the scientific literature.

Cationization of OA markedly increases the antigenicity of the protein and provides many more amino groups for EDC-catalyzed coupling of the  $E_2$ -3,4-Q-GABA complex. A solution of 50 mg OA was dissolved in 5 mL PB6.5 and dialyzed against PB6.5. The cationization reaction mixture included 2.0 mL OA solution, 200 mg of ethylenediamine HCl (EDHCl), 114 mg EDC, and 3.3 mg S-NHS. The mixture was stirred with a magnetic bar for 2 hr, at room temperature, and then dialyzed against PB6.5. A small volume of the catOA solution treated with an excess of EDC and S-NHS did not produce a precipitate, indicating efficient cationization of OA.

Production of the  $E_2$ -3,4-Q-GABA complex is unique to the present program. The reaction mixture included 57 mg GABA dissolved in 15 mL PB6.5 and 1.8 mL  $E_2$ -3,4-Q solution. The mixture was stirred for 2 hr, at room temperature and then extracted with chloroform. Unreacted  $E_2$ -3,4-Q was extracted into the organic phase as evidenced by its

yellow color. The red, aqueous layer containing the E<sub>2</sub>-3,4-Q-GABA complex, was evaporated to dryness. The dried powder was dissolved in 50% methanol (MeOH)/50% H<sub>2</sub>O, and unreacted GABA was removed with a one-gram reverse-phase cartridge (SepPak tC18). The red, cartridge effluent in 100% MeOH was evaporated to dryness and the purified E<sub>2</sub>-3,4-Q-GABA complex was stored as a dry powder.

Coupling E<sub>2</sub>-3,4-Q-GABA to catOA is unique to the present program. The coupling reaction mixture contained 3.0 mL catOA solution, all of the purified E<sub>2</sub>-3,4-Q-GABA prepared as described in the previous paragraph, 114 mg EDC, and 3.3 mg S-NHS. The mixture was stirred with a magnetic bar for 2 hr, at room temperature, and then dialyzed against PB6.5. The dialysis bag contained a red suspension that was separated by centrifugation into a deep red precipitate and a red supernatant. The latter was combined with cold acetone, lyophilized to a dry powder that exhibited a red color (E<sub>2</sub>-3,4-Q-GABA-catOA), and stored under vacuum. Spectral analysis of the soluble conjugate at 510 nm indicated that the molar ratio of E<sub>2</sub>-3,4-Q to catOA was 10:1. The red precipitate (more highly substituted E<sub>2</sub>-3,4-Q-GABA-catOA) was dried and then stored under vacuum.

#### B. Synthesis of a coating complex consisting of E<sub>2</sub>-3,4-Q covalently linked to bovine serum albumin (BSA)

##### 1. Rationale

The coating complex, a unique product of the present research initiative, was produced by direct reaction of E<sub>2</sub>-3,4-Q with BSA and not by coupling through the GABA linker. The complex serves in the enzyme-linked immunosorbent assay (ELISA) as an immobilized carrier of E<sub>2</sub>-3,4-Q epitopes that will be recognized by specific antibodies. These stable epitopes closely mimic stable E<sub>2</sub>-3,4-Q adducts that may form with intracellular or other proteins, thus selecting for antibodies that react well with endogenously formed adducts. In addition, since catOA and BSA exhibit little immunologic cross-reactivity, the use of catOA as immunogen and BSA as coating complex will enhance selection for antibodies of high specificity for E<sub>2</sub>-3,4-Q and selection against antibodies recognizing epitopes consisting of E<sub>2</sub>-3,4-Q and neighboring elements of the catOA surface. Thus, antibodies that bind with high affinity to E<sub>2</sub>-3,4-Q-BSA and do not bind to non-adducted BSA will effectively recognize stable adducts of E<sub>2</sub>-3,4-Q to any protein or other macromolecule.

##### 2. Synthesis of the coating complex, E<sub>2</sub>-3,4-Q-BSA

The coating complex was produced by direct reaction of E<sub>2</sub>-3,4-Q with BSA. The reaction mixture consisted of 11 mg of BSA dissolved in 2.5 mL of PB6.5 and dialyzed against PB6.5 and 300 µg of E<sub>2</sub>-3,4-Q dissolved in 300 µL of DMF. The reaction mixture was stirred at room temperature, for 15-20 hr. The conjugated protein was precipitated in cold acetone, washed 3 times with cold acetone to remove unreacted E<sub>2</sub>-3,4-Q, and then lyophilized. Spectral analysis of the conjugated protein indicated that the molar ratio of E<sub>2</sub>-3,4-Q to BSA was 5:1. The pink-colored, E<sub>2</sub>-3,4-Q-adducted protein was stored as a dry powder, under vacuum.

#### C. Synthesis of a low-molecular-weight competitor consisting of gamma-aminobutyric acid (GABA) linked *via* its primary amino group to carbon-1 of E<sub>2</sub>-3,4-Q

##### 1. Discussion

A published study (4) of the reaction of E<sub>2</sub>-3,4-Q with N-AcLys, a model compound containing a primary amino group, demonstrated a clean reaction producing E<sub>2</sub>-3,4-Q-1-N-AcLys and essentially no other linkage products (**Figure 2**). GABA is directly analogous to N-AcLys, consisting of a primary amino group separated by methylene groups from a carboxyl residue. Reaction of E<sub>2</sub>-3,4-Q with GABA and with N-AcLys proceeded at the same rate (change from yellow to red), and yielded products that yielded a single major band by HPLC analysis and exhibited the same absorption spectra with maxima at 510 nm. It is concluded that the product of the reaction between GABA and E<sub>2</sub>-3,4-Q is directly analogous to the product of the reaction between N-AcLys and E<sub>2</sub>-3,4-Q (**Figure 2**), and is E<sub>2</sub>-3,4-Q-1-N-GABA (**Figure 5**).

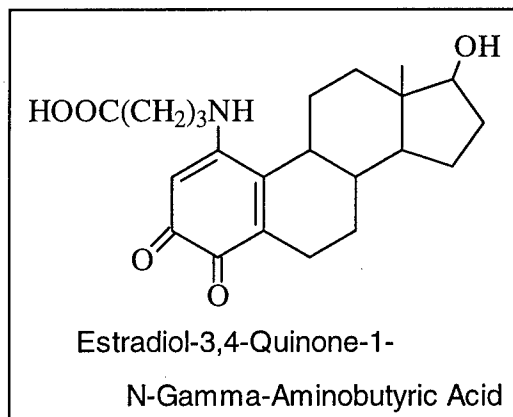


Figure 5

2. Synthesis of E<sub>2</sub>-3,4-Q-1-N-GABA is described in detail in Section 'A.4', above.
- D. Development of a novel enzyme-linked immunosorbent assay (ELISA) for detection, quantification, and immunochemical characterization of specific monoclonal antibodies
  1. Indirect ELISA for anti-E<sub>2</sub>-3,4-Q antibodies

The core of the ELISA is a novel coating complex developed as part of the present program (Section B) and consisting of E<sub>2</sub>-3,4-Q directly adducted to BSA. The operations are those of a conventional indirect ELISA. The assay was done with a 96-well immunoplate (Maxisorp; Nunc) some wells of which were coated with coating complex by exposure to 100  $\mu$ L of a solution consisting of 4 micrograms E<sub>2</sub>-3,4-Q-BSA per mL carbonate buffer at pH 9.6. Controls wells were coated by exposure to 100  $\mu$ L of a solution containing 4.5 micrograms BSA per mL carbonate buffer. Coated wells were washed with Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) and then treated with "blocking" buffer, a treatment that prevents nonspecific binding of primary antibody or enzyme-labeled secondary antibody. Blocked wells were washed and then exposed to primary antibody raised against the E<sub>2</sub>-3,4-Q epitope linked to protein, for 2 hr at 37 °C. Subsequently, unbound antibody was washed away and the wells were treated for 1 hr at 37 °C with horseradish peroxidase (HRP)-labeled secondary antibody specific for the primary antibody. Unreacted antibody was removed by washing with Tris-buffered saline (TBS). The wells were exposed to tetramethylbenzidine substrate for

20 min at room temperature, substrate conversion was stopped by addition of 1M H<sub>2</sub>SO<sub>4</sub>, and wells were read at 450 nm with an ELISA plate reader.

The indirect ELISA was applied to hybridoma screening, titration of anti-E<sub>2</sub>-3,4-Q antibodies, and in modified form, to estimating affinity of anti-E<sub>2</sub>-3,4-Q antibodies for the adducted quinone.

## 2. Competitive ELISA for anti-E<sub>2</sub>-3,4-Q antibodies

The competitive ELISA requires two unique components that are unique products of the present program; the coating complex (E<sub>2</sub>-3,4-Q-BSA) and the low-molecular-weight competitor (E<sub>2</sub>-3,4-Q-GABA). The competitive ELISA is implemented just as the indirect ELISA, with the exception of an additional operation that allows interaction of anti-E<sub>2</sub>-3,4-Q antibodies with a range of concentrations of the competitor, under conditions preserving the full functionality of the antibodies. The latter is fulfilled because of the water solubility of E<sub>2</sub>-3,4-Q-GABA in contrast to E<sub>2</sub>-3,4-Q which has very low solubility in aqueous media. In addition, the competitor provides an excellent mimic of E<sub>2</sub>-3,4-Q adducted to cellular and other proteins. Operationally, mixtures of primary antibody and different concentrations of E<sub>2</sub>-3,4-Q-GABA are incubated for 2 hr at 37 °C, prior to addition to the coated and blocked wells of the ELISA plate.

Inhibition of antibody binding to coated wells is calculated in reference to coated wells exposed to primary antibody, alone, and is expressed as % inhibition. Inhibition of antibody binding is plotted as a function of log E<sub>2</sub>-3,4-Q-GABA concentration. The reciprocal of the concentration of competitor at 50% inhibition is the affinity constant of the antibody.

## E. Production and selection of mouse hybridomas secreting antibodies specific for E<sub>2</sub>-3,4-Q adducted to proteins

### 1. Rationale

Mouse antibody-producing cells were induced and harvested according to a protocol that obviates long-term antigen-boosting and repeated sampling and testing of immune sera. A single bolus of immunogen in Freund's complete adjuvant is given subcutaneously and distal to the knee. Two weeks later, raised popliteal lymph nodes containing an array of lymphocyte clones reactive with antigen are easily removed and dissociated to single-cell suspensions for cell fusion. This timesaving approach depends on *ex corporeal* growth and selection of clones secreting high-affinity antibodies.

The first round of hybridoma production was based on immunization with E<sub>2</sub>-3,4-Q-OA having a low rate of haptentation (Section 'A.3', above). Antigen-reactive clones were rare and secreted IgM antibodies at low titers. It was concluded that induction of E<sub>2</sub>-3,4-Q-specific clones was poor because of the low rate of E<sub>2</sub>-3,4-Q substitution (2-3 molecules per molecule of OA) on the carrier protein. In addition, the high solubility of the minimally substituted carrier protein is expected to reduce the amount of immunogen taken up by antigen processing cells of the local lymph nodes.

The second and highly successful round of hybridoma production was based on immunization with cationized OA having a high rate of E<sub>2</sub>-3,4-Q substitution (Section 'A.4', above). The mice were given a primary immunization with soluble hapten-carrier complex having five times the haptentation rate as the previous hapten-carrier complex. Subsequently, the animals were given a single booster injection of a highly substituted

immunogen that remained a fine suspension in aqueous medium. The following section details the work with this protocol.

## 2. Production and selection of hybridomas secreting E<sub>2</sub>-3,4-Q-binding antibodies

Male Balb/c mice were given a single injection of soluble E<sub>2</sub>-3,4-Q-GABA-catOA (a novel immunogen developed as part of the present program) in Freund's complete adjuvant. Fifty microliters of inoculum containing 20 micrograms of immunogen were delivered subcutaneously in each hind foot/shank. Three weeks later, the mice were boosted at the same sites with 20 micrograms precipitated immunogen in Freund's incomplete adjuvant. Three days later, popliteal lymph nodes were harvested under sterile conditions and fused with non-secreting P3X63-Ag8.653 cells derived from a myeloma of Balb/c mice. The fusion products were cultured in 96-well plates, in growth medium supplemented with hybridoma cloning factor and a mixture of hypoxanthine, aminopterin, and thymine (HAT supplement). One week later, when clonal growth was observed, HAT supplement was replaced with hypoxanthine/thymine (HT) supplement. Culture medium was harvested when more than 50% of the bottom of a culture well was covered with growing cells. The culture was replenished with fresh medium. Harvested media were analyzed by an indirect ELISA (developed as part of the present program) for the presence of IGG antibody that produced a strong signal (= or > 1.0 absorbance unit) in E<sub>2</sub>-3,4-Q-BSA coated wells and only background signal (= or < 0.02 absorbance unit) in BSA coated wells. Ninety-one fusion cultures were screened and thirty-one met the criteria for expansion and further analysis. Six of the thirty-one candidates were exceptional, producing a specific signal = or > 2 and only background signal against BSA, alone. All candidates for further study were grown to high, cell density in 75 cm<sup>2</sup> T flasks and then cryopreserved.

Hybridoma Bd5 one of the six exceptional candidates was selected for cloning. One of these clones was evaluated for antibody production, and its MAb (MAb Bd5) was evaluated for selective binding to E<sub>2</sub>-3,4-Q-BSA, and affinity of binding to E<sub>2</sub>-3,4-Q-GABA a model of E<sub>2</sub>-3,4-Q adducted to proteins. Medium from T-flask cultures of clone Bd5 had a high titer of specific antibody, producing a strong binding signal at a dilution of more than 1000-fold (**Appendix One**). These same antibody preparations displayed absolute selectivity for the coating complex (E<sub>2</sub>-3,4-Q-BSA), producing a strong binding signal against coating complex and no signal against BSA, alone (**Appendix One**). The latter is an appropriate control for selective binding since the coating complex was produced by allowing E<sub>2</sub>-3,4-Q to adduct spontaneously to BSA in phosphate buffer at pH 6.5.

Antibody binding to the coating complex was studied by competitive ELISA with the water-soluble competitor E<sub>2</sub>-3,4-Q-GABA. A plot of log competitor concentration vs. % inhibition of antibody binding to the coating complex produced a classical sigmoid curve (**Appendix Two**). The antibody has an affinity constant of  $0.5 \times 10^8 \text{ M}^{-1}$  calculated from the IC<sub>50</sub> ( $2 \times 10^{-8} \text{ M}$ ) determined by competitive ELISA (**Appendix Two**). This calculation was based on the assumption that the concentration of free E<sub>2</sub>-3,4-Q-GABA was not significantly altered by antibody binding. According to these criteria, the antibody was determined to be an excellent reagent for immunochemical detection and quantification of the biological marker E<sub>2</sub>-3,4-Q adducted to cellular proteins or other macromolecules.

F. Development of novel immunohistochemical protocols for sensitive detection of E<sub>2</sub>-3,4-Q as a stable adduct to proteins and other macromolecules of breast epithelial cells collected in a non-invasive manner and fixed on slides, or tissue sections of breast tissue

1. Discussion

Protein coupling reactions (Section 'A.3' and Section 'B.2') and immunochemical assays (Section 'E.2') developed as part of the present program have established that 1) spontaneous formation of stable adducts of E<sub>2</sub>-3,4-Q to proteins *in vivo* is feasible, and 2) MAb Bd5 induced against E<sub>2</sub>-3,4-Q-catOA meets essential criteria for incorporation into immunohistochemical protocols for detection and quantification of E<sub>2</sub>-3,4-Q adducted to proteins or other macromolecules.

E<sub>2</sub>-3,4-Q spontaneously forms adducts to OA and BSA (as well as GABA) in phosphate buffer at pH 6.5. Molar ratios of adducted quinone to protein are 2:1 and 5:1, respectively. The basis for selective binding to a limited number of protein sites is unknown. Reaction of the quinone with protein is rapid and produces a distinctly red product that may be seen within 30 minutes after setting up the reaction mixture. The reaction between E<sub>2</sub>-3,4-Q and OA or BSA occurs at the same rate as that between the quinone and N-AcLys or GABA, and yields products with the same light absorbance. In view of published studies (4) indicating a high-yield, clean reaction between E<sub>2</sub>-3,4-Q and primary amino groups, it is concluded that E<sub>2</sub>-3,4-Q readily forms aminoquinone adducts to proteins.

T-flask cultures of hybridoma Bd5 produced high titers of MAb that bound specifically to E<sub>2</sub>-3,4-Q-BSA formed by spontaneous reaction of the quinone with BSA, and exhibited no detectable binding to BSA, alone (**Appendix One**). Competitive ELISA with an aminoquinone competitor produced by spontaneous adduction of E<sub>2</sub>-3,4-Q to GABA demonstrated high-affinity binding to the competitor (**Appendix Two**). Binding of the MAb to the coating complex (E<sub>2</sub>-3,4-Q-BSA) was inhibited 50% by competitor present at  $2 \times 10^{-8}$  M, indicating an affinity constant of  $0.5 \times 10^8 \text{ M}^{-1}$ , which is effective for immunohistochemical work.

Development of an immunohistochemical protocol for detection and quantification of E<sub>2</sub>-3,4-Q adducted to cellular proteins and other macromolecules required optimization of a set of 12-14 variables, some of which must be tested at multiple possible settings. This work was accomplished with E<sub>2</sub>-3,4-Q 'positive' sections of rat breast tissue and human MCF-7 cells (breast cancer cells) fixed to slides (analogous to nipple aspirate preparations). E<sub>2</sub>-3,4-Q 'positive' tissues and cells were produced by directly exposing tissues and cells to E<sub>2</sub>-3,4-Q at 2-10 picomols per mg solution, for 2 hours.

2. Immunohistochemical detection of stable E<sub>2</sub>-3,4-Q adducts in paraffin-embedded sections of breast tissue from ACI rats

Nearly all ACI female rats implanted with estradiol, develop breast tumors that are considered an animal model of human breast cancer (5). Work with this material was prompted by 1) the ready availability of paraffin-embedded sections of this material, 2) material demands of experimentation with a diversity of optimization conditions, and 3) the possibility of validating the protocol with a variety of samples from large repositories of paraffin-embedded breast tissue collected from humans. In addition, the results would provide an excellent starting point for work with breast epithelial cells fixed to slides (*e.g.*, nipple aspirate cells).

Protocol optimization studies yielded a great deal of information represented by the following selections. First, epitope recovery in hot citrate buffer destroyed the linkage of E<sub>2</sub>-3,4-Q to the primary amino group, and was eliminated from the protocol. Second, endogenous peroxidase activity in the tissue sections was high and required a peroxidase inhibition step in the protocol. Treatment of tissue sections with peroxide/methanol proved to be safe, preserving the integrity of adducted E<sub>2</sub>-3,4-Q and blocking endogenous peroxidase activity. Third, the primary antibody could be diluted as little as 1/50 without nonspecific background binding. Fourth, biotinylated secondary antibody produced strong and specific signals at a 1/5000 dilution. Fifth, it was necessary to incorporate a detergent into the wash solution. Sixth, it was possible to combine treatment with primary antibody and blocking of non-specific protein-binding sites in a single overnight treatment at 4°C. Seventh, methanol was the best solvent for delivery of the E<sub>2</sub>-3,4-Q-GABA competitor to primary antibody.

The immunohistochemical protocol included the following steps: 1) deparaffinize and hydrate tissue sections, 2) block endogenous peroxidase activity, 3) block endogenous avidin and biotin binding, 4) block nonspecific protein binding, 5) treat with primary antibody, 6) treat with biotin-labeled secondary antibody, 7) treat with horseradish peroxidase (HRP)-streptavidin, 8) treat with tetramethylbenzidine, 9) apply permount, and 10) observe sections, with a microscope.

The first critical test demonstrated intense, antibody binding site-dependent staining of ACI rat breast sections (formalin-fixed, paraffin-embedded) treated briefly with E<sub>2</sub>-3,4-Q in phosphate buffer (after deparaffinization and hydration of the sections), and then processed with the immunohistochemical protocol (**Appendix Three**). Both of two paired sections on a single slide were exposed to a solution of 5-10 picomols E<sub>2</sub>-3,4-Q per mg solution (mimicking concentrations of 4-OHE<sub>2</sub> found in human breast tumors). One section was exposed to MAb treated with methanol carrier (**Appendix Three, panels A and C**) and the paired section was treated with MAb exposed to  $2 \times 10^{-7}$  M E<sub>2</sub>-3,4-Q-GABA in methanol (**Appendix Three, panels B and D**). It is estimated that only a small fraction of a per cent of the E<sub>2</sub>-3,4-Q became adducted to the tissue.

The second critical test demonstrated antibody-dependent, E<sub>2</sub>-3,4-Q-specific staining of sections of ACI rat breast (**Appendix Four**). One of two paired sections was exposed to 5-10 picomols E<sub>2</sub>-3,4-Q per mg solution (**Appendix Four, panels A and C**) and the second section was exposed to 300-600 picomols of E<sub>2</sub>-2,3-Q per mg solution (**Appendix Four, Panel D**). Both sections were exposed to untreated MAb Bd5. The slide was then processed with the immunohistochemical protocol. Panels 'A' and 'C' exhibited intense staining with tetramethylbenzidine, and panels 'B' and 'D' exhibited no staining.

### 3. Immunohistochemical detection of stable E<sub>2</sub>-3,4-Q adducts in human MCF-7 cells attached to microscope slides

The protocol described in Section 'F.2' required significant revision for application to whole cells attached to microscope slides. Some of these are described as follows. First, cells were air-dried onto glass slides and fixed with 95% ethanol. Second, air-dried cells required rehydration in buffer and a brief fixation with glutaraldehyde. The latter prevented significant loss of protein caused by required washings with detergent and buffer. Third, the nuclear and cytoplasmic membranes of intact cells fixed to slides pose significant barriers to primary and secondary antibodies as well as to HRP-streptavidin. Consequently, the revised protocol required membrane permeabilization with acetone at



-15 °C. In addition, peroxidase, biotin, and avidin blocking steps were found unnecessary.

The revised immunohistochemical protocol included the following steps: 1) air-dry and fix cells onto glass slides, 2) hydrate cells and stabilize protein with glutaraldehyde, 3) permeabilize cytoplasmic and nuclear membranes with cold acetone, 4) block nonspecific protein binding, 5) treat with primary antibody, 6) treat with biotin-labeled secondary antibody, 7) treat with HRP-streptavidin, 8) treat with tetramethylbenzidine, 9) apply permount, and 10) observe sections, with a microscope.

A critical test was done with cytospin preparations of normal MCF-7 cells from the same cell suspension (**Appendix Five**). The cell spots on each of two slides were fixed with glutaraldehyde and then exposed to 1-2 picomols E<sub>2</sub>-3,4-Q per mg buffer solution, for 2 hours. The spot on one slide was exposed to MAb treated with methanol carrier (**Appendix Five, panels A and C**) and the paired spot on the second slide was treated with MAb exposed to 10<sup>-7</sup>M E<sub>2</sub>-3,4-Q-GABA in methanol (**Appendix Five, panels B and D**). Again, it is estimated that only a small fraction of a per cent of the E<sub>2</sub>-3,4-Q became adducted to the tissue. The test indicated strong, specific staining of E<sub>2</sub>-3,4-Q treated cells.

#### G. Tissue fixation issues

A recent test indicated that formaldehyde and glutaraldehyde destroy the linkage of E<sub>2</sub>-3,4-Q to the primary amino group. This test was done in anticipation of validation studies with benzanthracene- or dioxin-induced MCF-7 cells characterized by markedly increased expression of the estrogen 4-hydroxylase Cyp1B1. In this case, adducts of E<sub>2</sub>-3,4-Q to proteins and other macromolecules are expected to form endogenously, during cell culture. Preliminary tests with acetone fixation of MCF-7 cells previously exposed to exogenous E<sub>2</sub>-3,4-Q and then stained with Mab Bd5 demonstrated antibody-dependent staining (**Appendix Six, panel A**). Control cells exposed to exogenous E<sub>2</sub>-3,4-Q and then stained with MAb Bd5 blocked with E<sub>2</sub>-3,4-Q-GABA, did not exhibit TMB staining (**Appendix Six, panel B**). Cellular integrity, however, was compromised as manifested by loss of nuclear delineation and reduced staining intensity. Revised tissue fixation protocols that will stabilize cellular proteins and preserve the integrity of the E<sub>2</sub>-3,4-Q epitope must be developed and tested. An effective tissue-fixation protocol is fundamental to studies aimed at validating the application of MAb Bd5 to detection of endogenously formed E<sub>2</sub>-3,4-Q adducts to tissue proteins as a biological marker of E-DC.

#### KEY RESEARCH ACCOMPLISHMENTS

- ◆ Demonstrated that estradiol-3,4-quinone (E<sub>2</sub>-3,4-Q) spontaneously adducts to primary amino groups of proteins and small molecules, *e.g.*, gamma-aminobutyric acid (GABA) and *N*-acetyl-*L*-lysine, near physiological pH, supporting the possibility of endogenous formation of stable adducts of E<sub>2</sub>-3,4-Q to proteins, *in vivo*
- ◆ Developed reaction conditions for producing a novel E<sub>2</sub>-3,4-Q-specific 1) immunogen, 2) coating complex, and 3) competitive inhibitor
- ◆ Developed a novel linker-adduct conjugate by coupling E<sub>2</sub>-3,4-Q to GABA, and purified the conjugate by a combination of chloroform extraction, phase separation during rotary evaporation, and solid-phase extraction with a Sep-Pak cartridge

- ◆ Developed a novel immunogen for induction of B cell clones specific for E<sub>2</sub>-3,4-Q, by over-coupling the linker-adduct conjugate E<sub>2</sub>-3,4-Q-GABA to hen egg albumin (OA), in the presence of the carboxyl activator 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxy-sulfosuccinimide (S-NHS) which forms a stable ester intermediate with the activated carboxyl group
- ◆ Developed a coating complex for enzyme-linked immunosorbent assay (ELISA), by directly reacting E<sub>2</sub>-3,4-Q with bovine serum albumin (BSA) thereby producing a physiologically relevant conjugate
- ◆ Developed an indirect ELISA and a competitive ELISA for hybridoma screening and immunochemical characterization of candidate MAbs
- ◆ Developed a high-affinity MAb (Bd5) specific for E<sub>2</sub>-3,4-Q adducted to any protein
- ◆ Demonstrated efficient and specific immunohistochemical staining of formalin-fixed or glutaraldehyde-fixed tissues exposed to exogenous E<sub>2</sub>-3,4-Q at low picomole quantities
- ◆ Determined essential conditions for development of tissue processing required for immunohistochemical detection of stable E<sub>2</sub>-3,4-Q adducts formed endogenously in target tissues

#### REPORTABLE OUTCOMES

A patent disclosure entitled "Stable Adducts of Estradiol-3,4-Quinone to Proteins and Other Macromolecules As A Potential Biomarker of Breast Cancer Risk: Monoclonal Antibody-Based Detection and Quantification in Nipple Aspirate Cells" is under consideration by representatives of the "Intellectual Property Office" of the University of Nebraska Medical Center.

#### CONCLUSIONS

Work supported by the present "Concept Award" has produced and characterized a novel MAb (Bd5) exhibiting high specificity and affinity for E<sub>2</sub>-3,4-Q adducted to any protein. In the indirect ELISA, the antibody produced a strong signal against E<sub>2</sub>-3,4-Q-BSA and no signal against BSA, alone. As determined by competitive ELISA, the reaction between the MAb and E<sub>2</sub>-3,4-Q-GABA 1) yielded a classical bimolecular plot of E<sub>2</sub>-3,4-Q-GABA concentration vs. reduction of ELISA signal, 2) exhibited saturation (100% inhibition of the ELISA signal) at *ca.*  $2 \times 10^{-7}$  M E<sub>2</sub>-3,4-Q-GABA, and 3) had a *K<sub>a</sub>* of  $0.5 \times 10^8$  M<sup>-1</sup>. Hybridoma Bd5 secreted the MAb in relatively high abundance as evidenced by the high dilution (1000 to 2000) of T flask culture medium that produced a strong signal against E<sub>2</sub>-3,4-Q-BSA in the indirect ELISA. MAb-based immunohistochemical staining produced a strong signal in tissues treated with a few picomoles of E<sub>2</sub>-3,4-Q and no signal in tissues treated with a few hundred picomoles of E<sub>2</sub>-2,3-Q, indicating effective discrimination of the two estrogen quinones.

MAb Bd5 is a novel probe that offers the opportunity to examine the relationship between non-detoxified E<sub>2</sub>-3,4-Q (as evidenced by presence and abundance of stable adducts to proteins) in cells prepared from nipple aspirates or fine-needle aspirates of the breast, and breast cancer risk. This can be achieved through implementation of technologies such as Quantitative Fluorescence Imaging Analysis (QFIA) (6) or Surface-Enhanced Laser Desorption Ionization/tandem mass spectrometry (SELDI/MS/MS) (7) for quantification in individual cells, of E<sub>2</sub>-3,4-Q adducts to proteins or E<sub>2</sub>-3,4-Q-

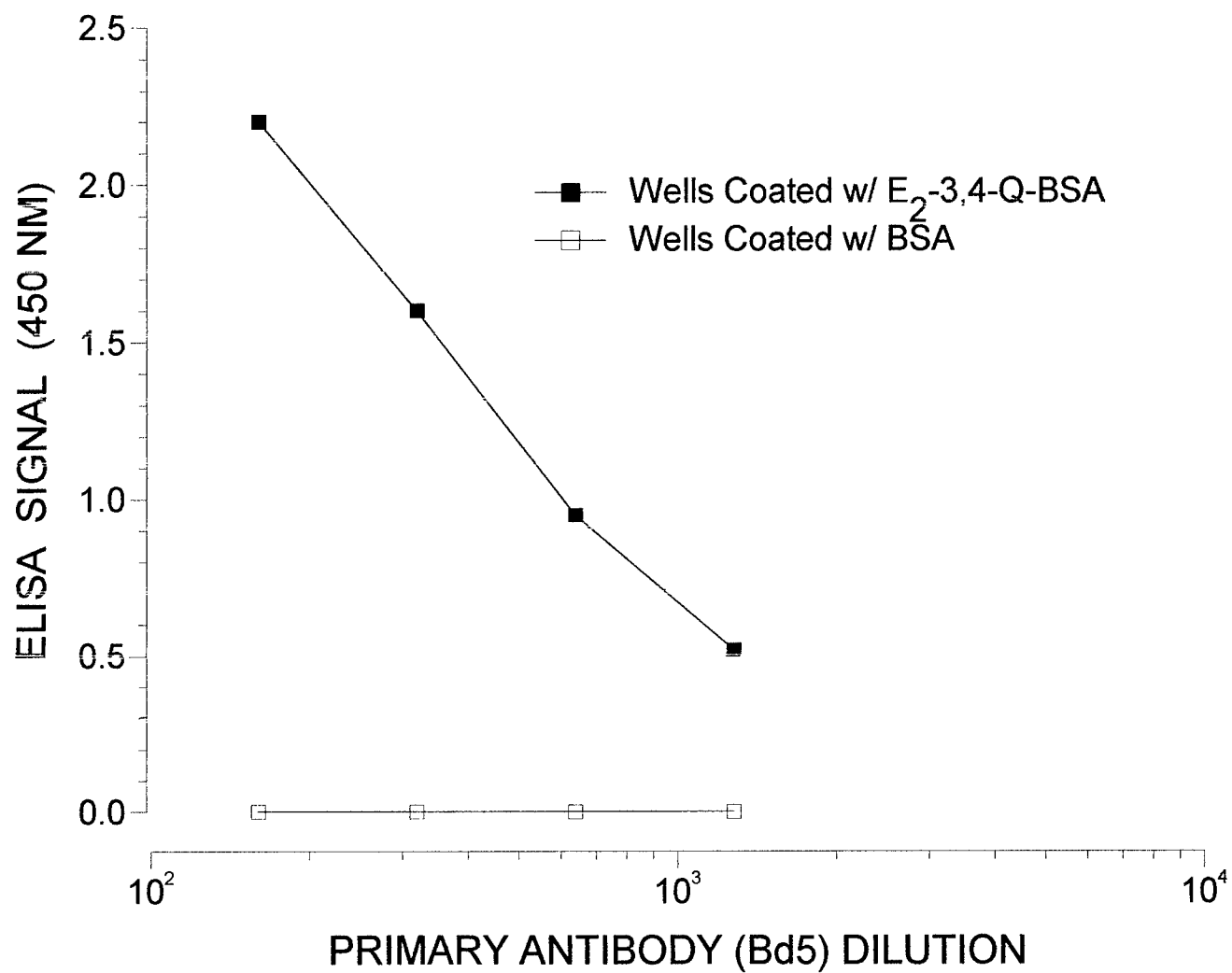
adducted proteins, respectively. QFIA is potentially capable of detecting approximately 100 molecules of adducted protein per cell. MAb Bd5 will make possible acquisition of information needed to determine whether post-translational modification of proteins by non-detoxified E<sub>2</sub>-3,4-Q is an important biomarker of breast cancer risk. If the latter is established, the antibody will serve as the keystone of protocols for determining breast cancer risk and efficacy of preventive interventions that may be based on reducing non-detoxified E<sub>2</sub>-3,4-Q in breast cells.

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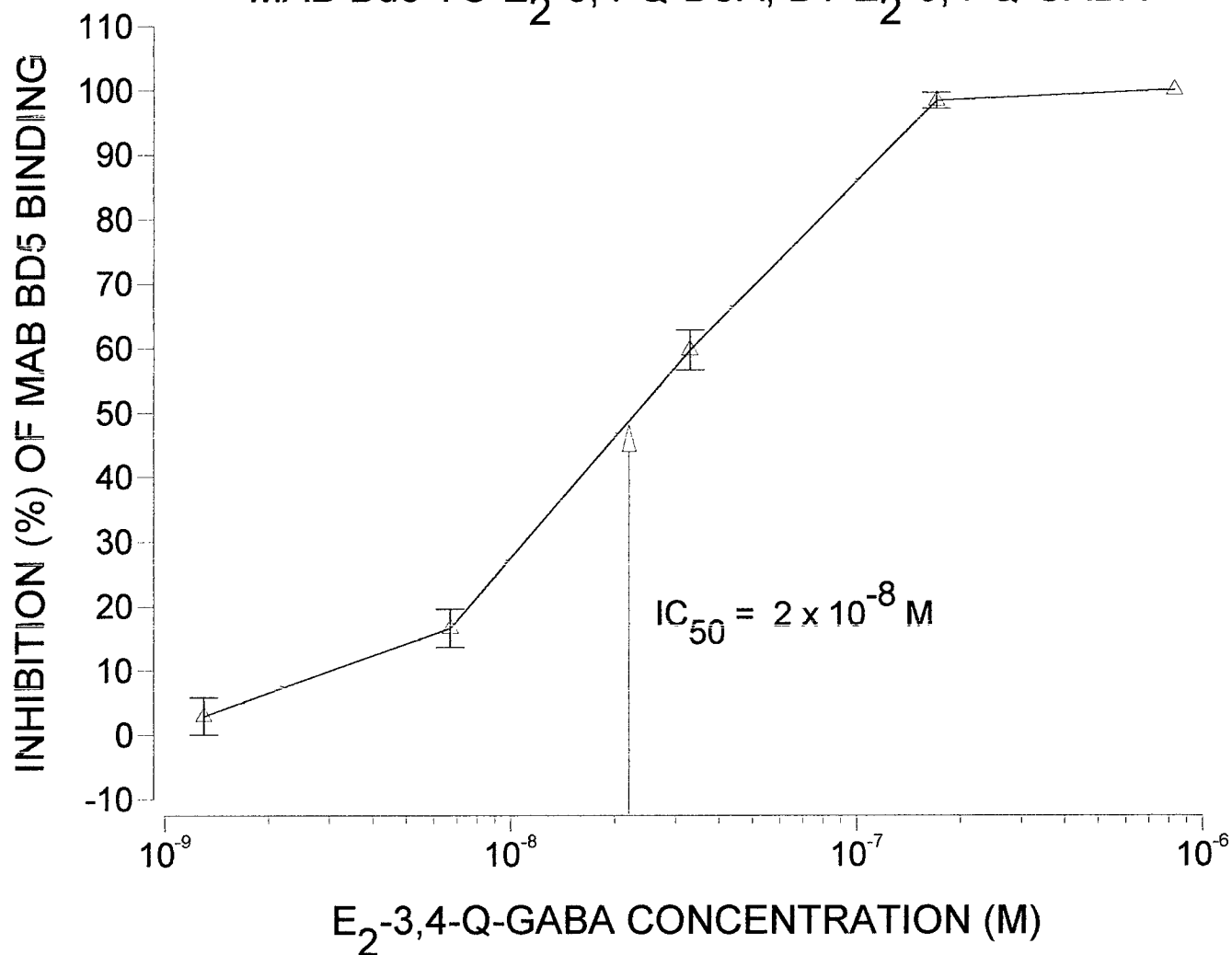
## APPENDIX ONE

### SPECIFIC BINDING OF MONOCLONAL ANTIBODY Bd5 TO ESTRADIOL-3,4-QUINONE-BSA, DETERMINED BY INDIRECT ELISA WITH AN HRP-ANTI-MOUSE IGG ANTIBODY

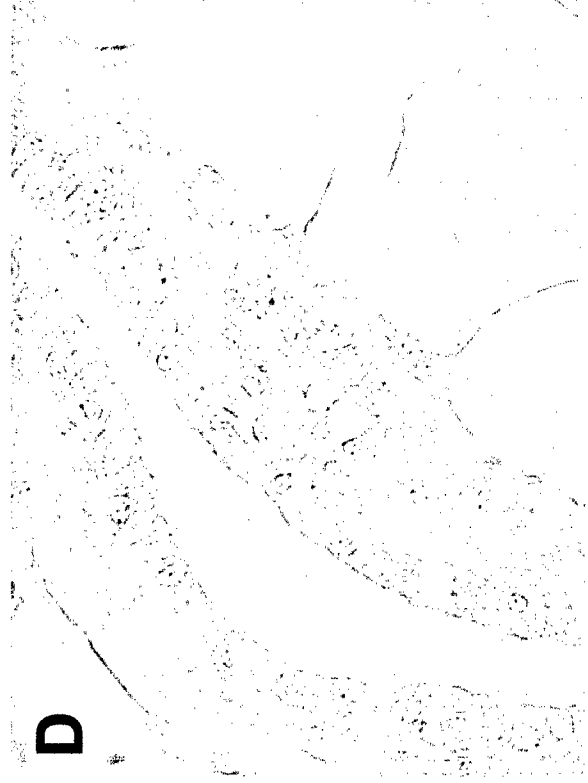
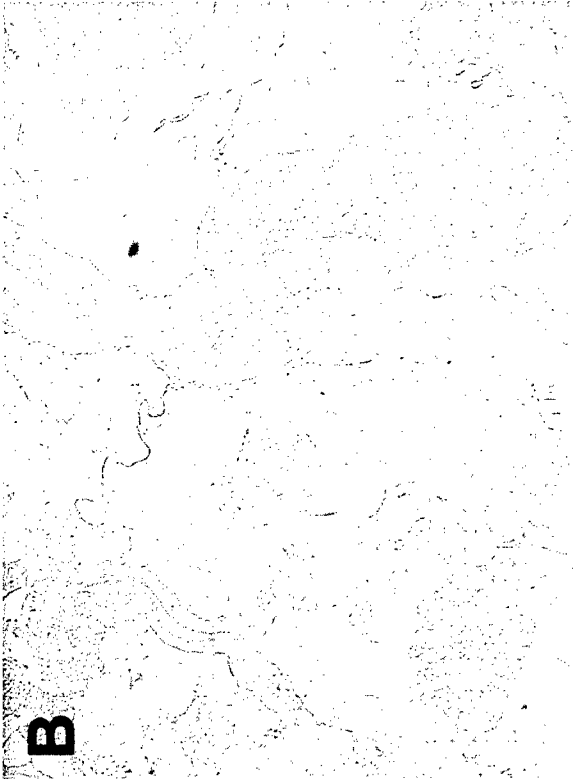
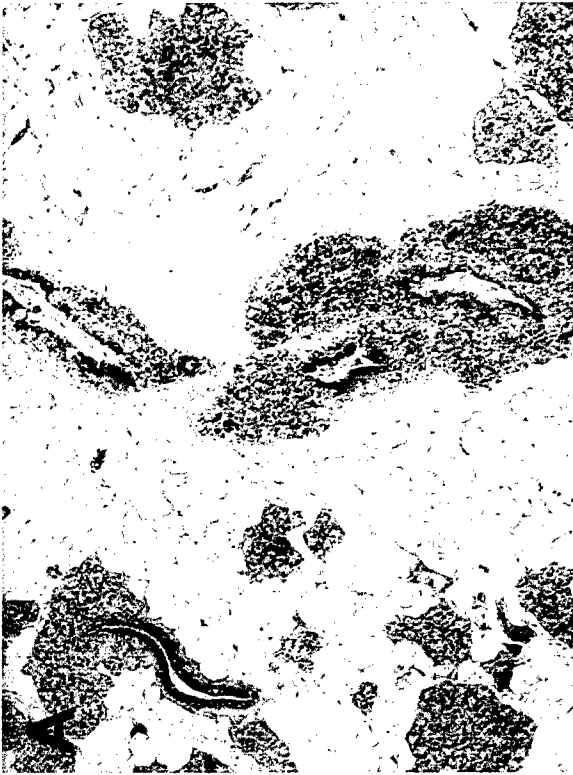


## APPENDIX TWO

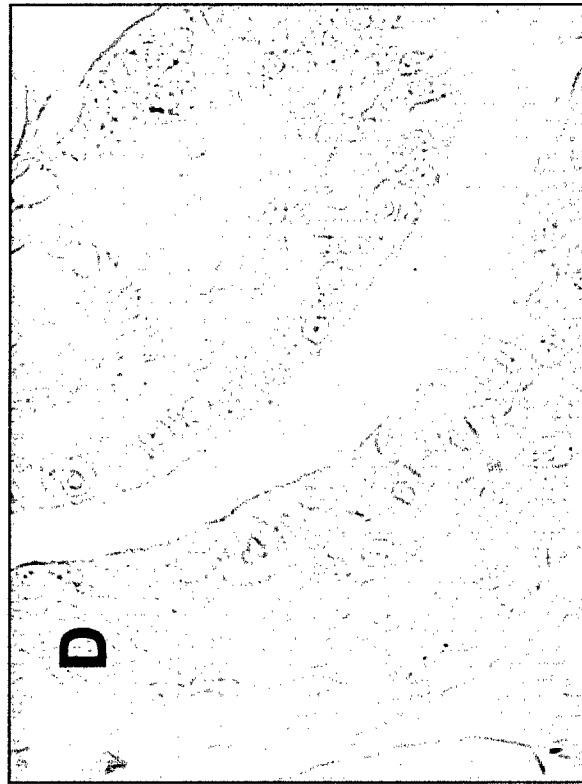
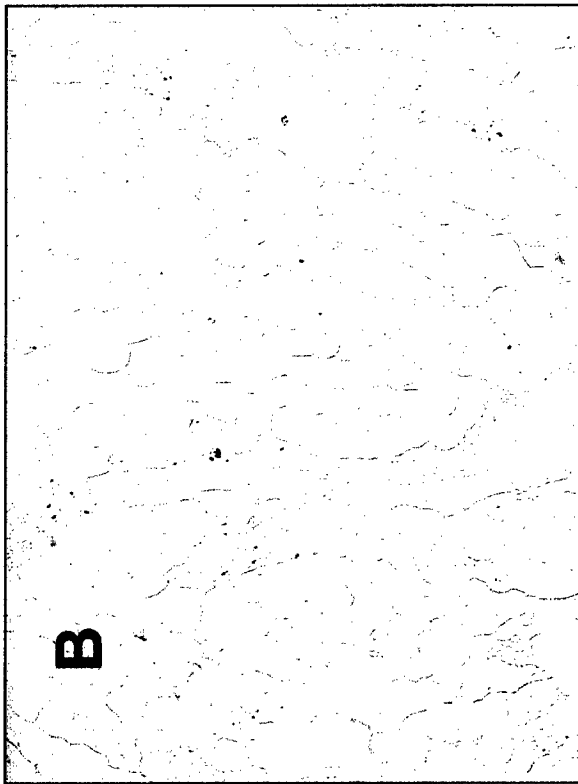
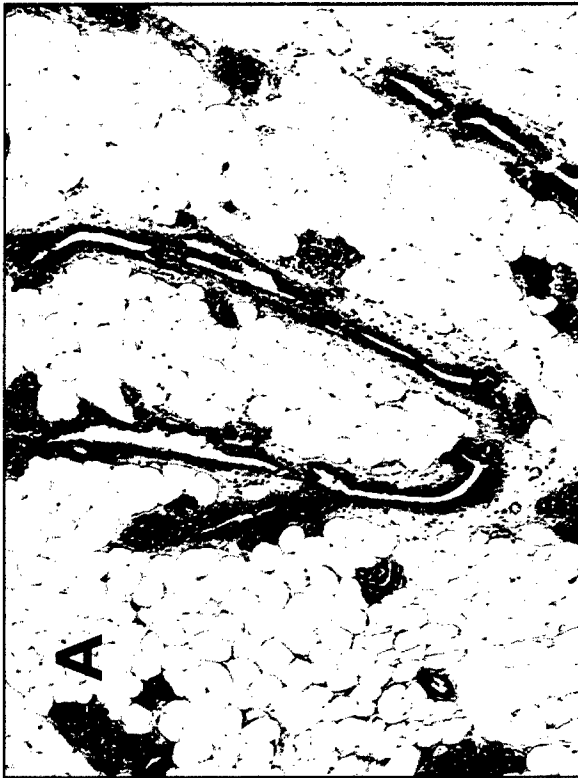
### COMPETITIVE ELISA: INHIBITION OF SPECIFIC BINDING OF MAB Bd5 TO E<sub>2</sub>-3,4-Q-BSA, BY E<sub>2</sub>-3,4-Q-GABA



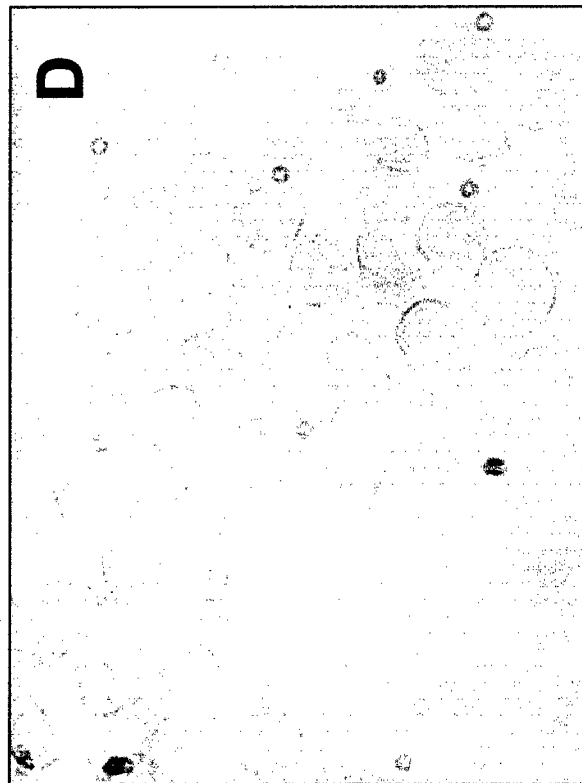
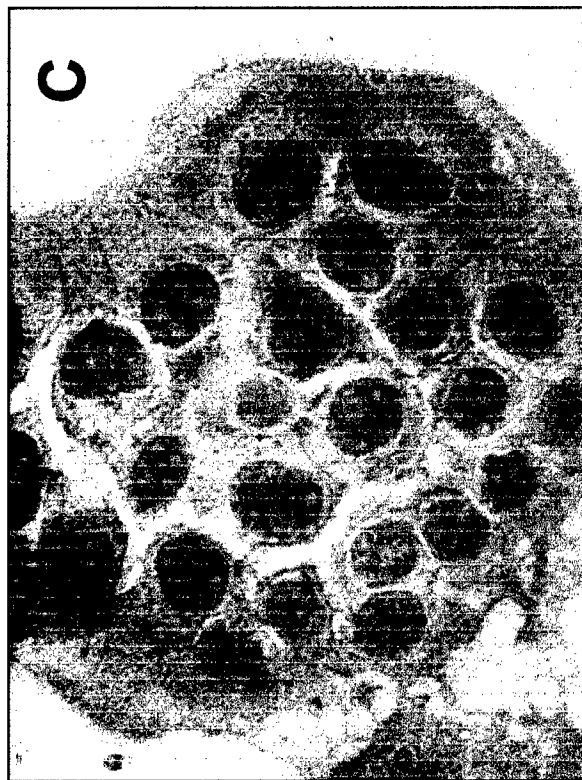
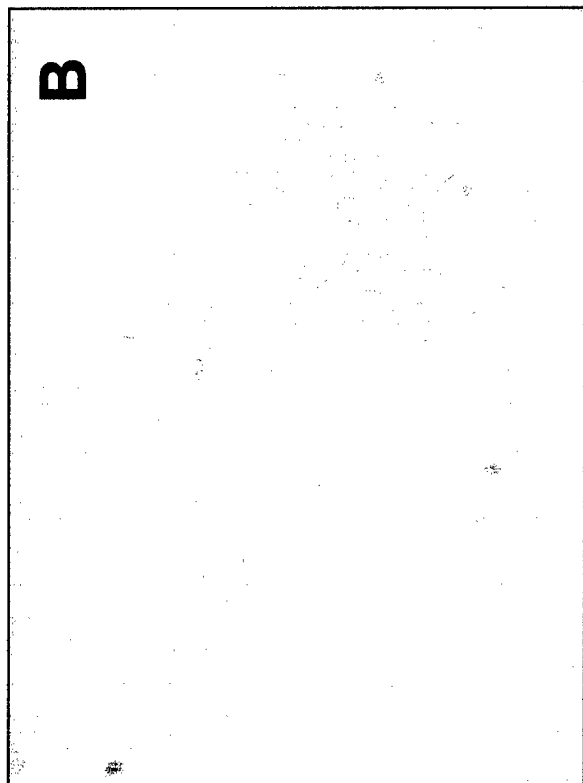
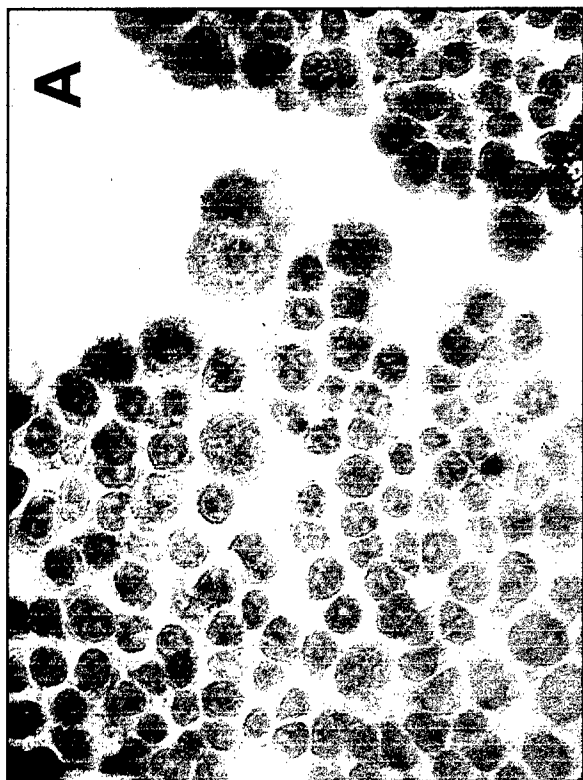
### APPENDIX THREE



APPENDIX FOUR

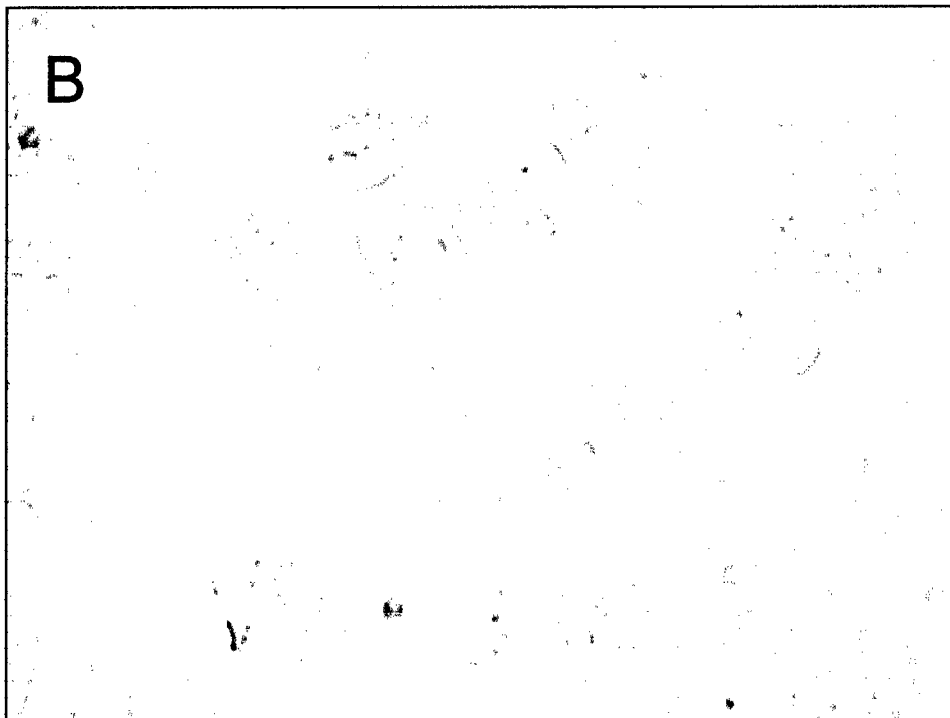
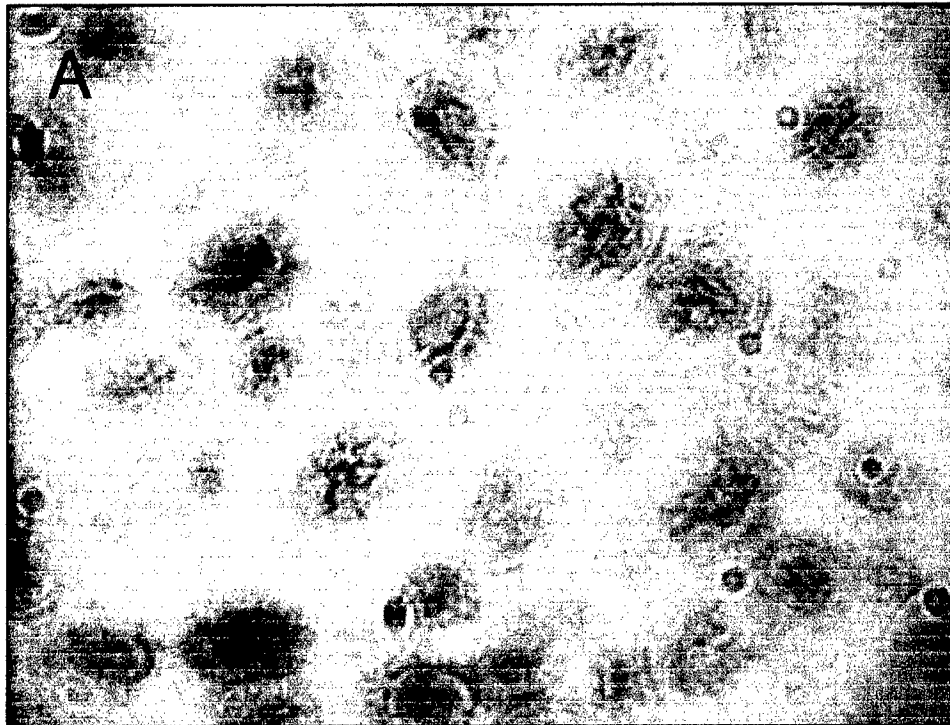


APPENDIX FIVE





APPENDIX SIX





DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

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11 Mar 03

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

A handwritten signature in cursive script, reading "Phyllis Rinehart", is positioned above the typed name.

PHYLLIS M. RINEHART

Deputy Chief of Staff for  
Information Management

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ADB282172  
ADB261548  
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